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# The Versatile Roles of "Axon Guidance" Cues in Tissue Morphogenesis

Review

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**The Netrin, Slit, Semaphorin, and Ephrin families of secreted proteins were originally characterized in the nervous system as guidance cues for axons; however, recent studies demonstrate that many members of these families contribute to the development of a variety of organs. Here, the current knowledge of their roles is discussed with a focus on four tissues: lung, mammary, cardiovascular, and kidney. While many studies indicate a role for "axon guidance" cues in regulating cell-cell and cell-extracellular matrix (ECM) interactions during organogenesis, there is accumulating evidence that they also contribute to tissue development by regulating the transcription and translation of genes encoding key morphogenetic factors.**

After the three germ layers of an embryo are established in development, organogenesis begins as cells interact with each other and arrange themselves into tissues and organs. In the nervous system, guidance cues organize neural progenitor cells and direct axons into intricate networks of connections. There is a growing list of secreted proteins, including morphogens such as Wnts (Zou, 2004), that act as neuronal guidance cues. This review focuses on four families (Netrin [*Ntn*], Slit [*Slit*], Semaphorin [*Sema*], and Ephrin [*Efn*]) whose axon guidance activities have been extensively studied, but whose functions outside the nervous system are just beginning to be elucidated. These cues are present in the extracellular environment and are either expressed on the cell surface or secreted into the ECM, where they are thought to form gradients that affect neuronal behavior at long range. They can act as attractants, guiding neurons and their axons to targets, or repellents, creating exclusion zones that neurons avoid. Thus, the range and mode of action of these cues established their original classifications: Ephrins and glycosylphosphatidylinositol (GPI)-linked Semaphorins as short-range repellents; Slits and secreted Semaphorins as long-range repellents; and Netrins as long-range bifunctional cues, capable of both attraction and repulsion. But as an increasing number of activities for these cues are discovered, stringent labels defining their function are no longer appropriate. For example, Slits act as attractants and Ephrins promote postsynaptic receptor clustering (Kramer et al., 2001; Palmer and Klein, 2003). Furthermore, there are an increasing number of reports describing the expression of these cues outside the nervous system in a variety of developing tissues and organs. In some settings, their functions can be defined by analogous roles in the nervous system. In other settings, evidence suggests they govern tissue morphogenesis in new ways that are not simply defined in terms of their neuronal guidance functions. Here, current information is reviewed on the roles these axon guidance cues play during organogenesis with a focus on four developing tissues: lung, mammary, cardiovascular, and kidney.

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## Guidance Cues and Their Receptors

The response of a cell to a guidance cue depends on specific receptors expressed on the cell surface. Many of these receptors have been identified and their domain structure determined (Figure 1). There are some structural similarities between receptors; for example, both Netrin and Slit receptors are members of the immunoglobulin superfamily, but for the most part the cues and their receptors are not structurally related. These differences translate into unique signaling strategies such as different ways in which coreceptors are employed. Class 3 Semaphorins have ligand binding coreceptors, called Neuropilins (*Nrp1* and *Nrp2*), that act together with a family of signal-transducing proteins, called Plexins (*Plxn*) (Tamagnone and Comoglio, 2000). Netrins also have two families of receptors: the DCC (Deleted in Colorectal Cancer) family comprising Dcc (*Dcc*) and Neogenin (*Neo1*) and the UNC5 family comprising *Unc5a*, *Unc5b*, *Unc5c*, and *Unc5d* (Engelkamp, 2002; Livesey, 1999). Members of the DCC and UNC5 family both bind ligand and transduce independent intracellular signals (Livesey, 1999). They also bind each other, acting as coreceptors for Netrin-dependent repulsion (Hong et al., 1999). Another unique signaling strategy is bidirectional signaling employed by transmembrane Ephrins and their Eph (*Eph*) receptors (Palmer and Klein, 2003). Bidirectional signaling is different from traditional signaling between ligand and receptor in which ligand induces intracellular events only within receptor-expressing cells. In bidirectional signaling, intracellular changes can occur in both receptor-expressing cells (forward signaling) and ligand-expressing cells (reverse signaling), since the transmembrane ligand and its receptor can both send as well as receive signals. Of all the receptors that transduce guidance signals, only one, the Eph kinase receptor, has intrinsic enzymatic activity. The others rely on a variety of adaptor proteins to link them to signaling systems (Huber et al., 2003).

Despite these differences, all guidance factors direct the motility of cells and, in each case, this appears to be accomplished by a receptor interacting, directly or indirectly, with a member of the Rac family of small GTPases (Huber et al., 2003). These effector proteins in turn organize the response of receptor-expressing cells by regulating the structure and dynamics of the actin cytoskeleton. A second common feature of these cues facilitates the regulation of cell movement. Each cue interacts with its receptor with a dissociation constant in the nanomolar range. This is different from the picomolar dissociation constants that characterize many ligand/receptor interactions such as the interaction between

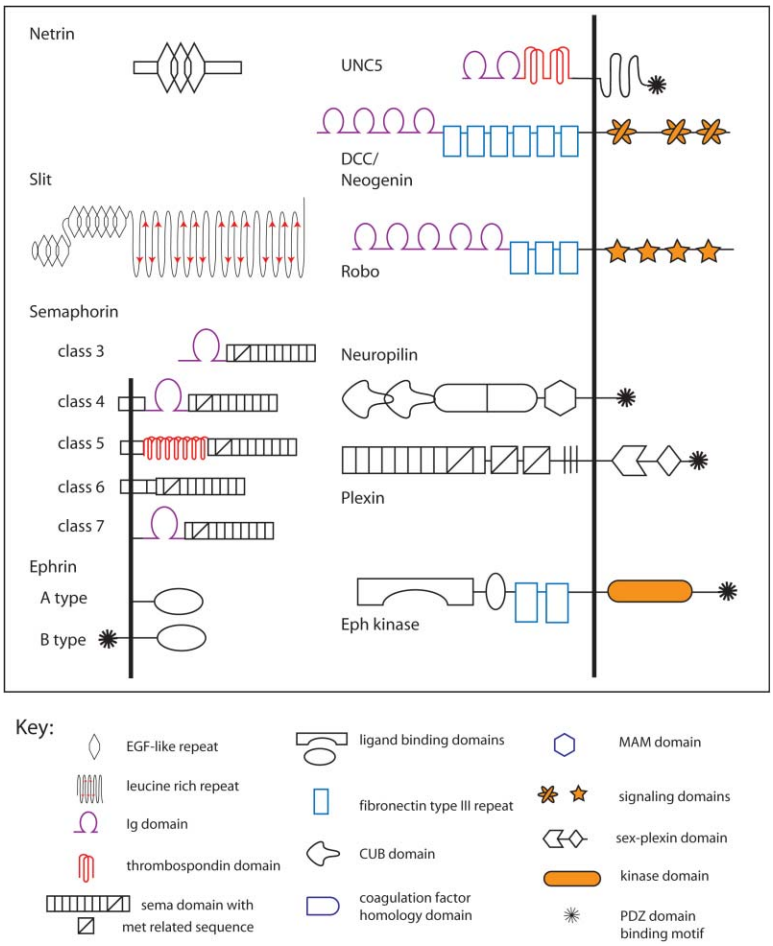


Figure 1. Domain Structure of Guidance Cues and Their Receptors

Members of a family of guidance cues may be secreted from the cell or tethered to the membrane via GPI or transmembrane linkages. Receptors are all single-pass transmembrane proteins. Abbreviations: EGF, epidermal growth factor; Ig, immunoglobulin; CUB, complement-homology domain; MAM, meprin/A5/mu-phosphatase homology domain; PDZ, motif that was originally discovered in three proteins, PSD-95, Dlg1, and ZO-1.

EGF and the EGF receptor (Hurwitz et al., 1991). Interactions of this affinity apparently permit the rapid sampling of the environment observed when a growth cone actively explores its surroundings. Filopodia are extended that make and release contacts as the growth cone follows gradients of secreted cues present in the ECM. In thinking about the function of these cues during tissue morphogenesis, it is possible that such flexible interactions between receptor and cue permit active, yet constrained, mobility of cells during morphogenetic remodeling. Since these interactions between cue and receptor range from adhesive/attractive to anti-adhesive/repulsive and occur between individual cells and between cells and their environment, they could allow for wide-ranging morphological effects; for example, they could participate in molding and shaping living tissues in 3-dimensional space. It is therefore not surprising that, given such a potential for broad-spectrum activity, guidance cues are employed in many contexts throughout development.

## Lung Branching Morphogenesis: Push-Pull Mechanism of Netrins and Semaphorins

The mammalian lung exemplifies an architectural transformation as it grows from a simple epithelial bud into a complex tree-like structure designed for gas exchange. Members of all four families of guidance cues are expressed during lung development (Anselmo et al., 2003; Greenberg et al., 2004; Hafner et al., 2004; Xian et al., 2001), but functional studies relate the activity of only two families, Semaphorin and Netrin, to specific morphogenetic events (Ito et al., 2000; Kagoshima and Ito, 2001; Liu et al., 2004). One current hypothesis is that guidance cues establish permissive and restrictive zones during early steps of branching morphogenesis. The budding epithelium responds to these zones by refining the size and shape of the outgrowth, an activity similar to the role these cues play during neural development when they shape the architecture of the nervous system by directing the outgrowth of axons.

Murine lung development begins at embryonic day 9 when two primordial buds composed of an inner endodermal epithelium and an outer mesenchymal jacket grow out of the primitive trachea (Cardoso, 2000). In the first stage of lung development, the bronchial tree develops through a process of elongation, branching, and budding, giving rise to four bronchial stems on the right and one on the left. Development continues as dichotomous branching establishes the conducting portion of the airways and generates terminal bronchioles. Terminal bronchioles eventually give rise to primitive alveolar ducts that end in terminal sacs, and these ultimately develop into mature alveolar ducts and alveoli that compose the adult lung.

Current evidence suggests that SEMA3A, SEMA3C, NTN1, and NTN4, along with their respective receptors,

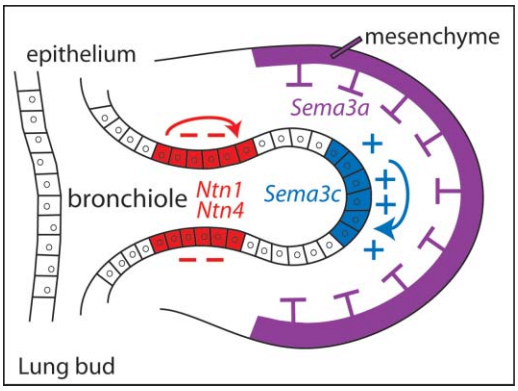


Figure 2. The Expression Patterns of Semaphorins, Netrins, and Their Receptors along with Their Attractive versus Repulsive Functions May Create Push-Pull Forces that Shape the Architecture of a Lung Bud

In this model, Netrins function proximally to restrict ectopic budding. *Ntn1* and *Ntn4* transcripts are expressed together in the stalk region (red), and the proteins encoded by these transcripts are deposited into the basal lamina surrounding the bud stalk (red −). Transcripts of the components of the repellent receptor complex, *Unc5b* and *Dcc*, are expressed in an overlapping manner in the stalk epithelium and are present to receive this restrictive cue (red). Transcripts of *Sema3a* are expressed distally in the mesenchyme and function to fine-tune the size and shape of emerging buds (purple). Transcripts of the SEMA3A receptor, *Nrp1*, are present to receive this restrictive signal in the epithelium of terminal buds (blue). These repulsive activities are balanced by the attractive function of SEMA3C, transcripts of which are expressed in the epithelium of terminal buds (blue), and the protein encoded by these transcripts is deposited into the basal lamina surrounding the terminal bud (blue +). To receive this positive cue, transcripts of the SEMA3C receptor, *Nrp2*, are expressed in an overlapping manner with the transcripts of *Sema3c* in the epithelium of terminal buds (blue). Figure from Kagoshima and Ito (2001).

play a role in defining the pattern of early branching morphogenesis (Ito et al., 2000; Kagoshima and Ito, 2001; Liu et al., 2004). From E11.5 to E13.5, these cues are expressed in overlapping patterns as the respiratory tree develops (Figure 2). *Sema3c* transcripts are expressed in the epithelium at the tips of the bronchioles, while *Sema3a* transcripts are expressed in a complementary pattern in the surrounding mesenchymal jacket (Ito et al., 2000; Kagoshima and Ito, 2001). *Ntn1* and *Ntn4* transcripts, like *Sema3c*, are expressed together in the epithelium, but rather than being expressed at the tips of the bronchi buds, Netrin transcripts are localized to the stalk regions (Liu et al., 2004). All these cues transduce signals through receptors that are present in the epithelium.

Several of these cues, SEMA3A, NTN1, and NTN4, have been shown to act repulsively in the nervous system and may have a similar function in the lung. Transcripts of *Nrp1*, which encode a receptor that mediates SEMA3A-dependent repulsion in the nervous system (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997), are present in terminal buds adjacent to *Sema3a*-expressing mesenchyme (Kagoshima and Ito, 2001). Transcripts of *Unc5b*, which encode a Netrin receptor, are expressed along with a second Netrin receptor DCC in the epithelium (Liu et al., 2004). Together, these receptors mediate repulsion in a Netrin-dependent manner (Hong et al., 1999). Given these patterns of expression, one hypothesis concerning the function of these cues is they work in concert to restrict ectopic budding and fine-tune both the size and shape of emerging buds. In this model, NTN1 and NTN4, deposited in the basal lamina surrounding the bud stalks, function in the proximal region of the duct to prevent inappropriate lateral branching. In contrast, SEMA3A, present in the mesenchyme surrounding the end of buds, acts distally to organize their shape (Figure 2). Results from in vitro assays, showing that these cues inhibit branching, support this model (Ito et al., 2000; Liu et al., 2004). In the case of NTN1 and NTN4, this inhibition is so dramatic that buds project internally into the lumen rather than externally to form new projections (Liu et al., 2004).

These inhibitory activities could be balanced by the chemoattractant action of SEMA3C (Bagnard et al., 1998; de Castro et al., 1999), whose transcripts are expressed in the epithelium at the tips of the bronchioles (Figure 2; Kagoshima and Ito, 2001). In the model, transcripts of *Nrp2*, which encode a receptor that binds SEMA3C (Chen et al., 1997; Takahashi et al., 1998), are present in the epithelium and could receive this signal (Kagoshima and Ito, 2001). In the model, SEMA3C counters the repellent effects of SEMA3A, NTN1, and NTN4 and stimulates budding in appropriate locations. Indeed, treatment of lung explants with SEMA3C demonstrates its stimulatory effect on branching morphogenesis, resulting in a more highly branched structure and a modest increase in cell proliferation (Kagoshima and Ito, 2001).

Taken together, it is possible to generate a push-pull model of lung branching morphogenesis whereby guidance cues act in concert with other critical growth and morphogenetic factors to sculpt the architecture of the respiratory tree (Figure 2). It is, therefore, disappointing that results generated by treating lung explants with purified forms of guidance cues are not complemented by loss-of-function phenotypes in knockout mice. There are no morphological abnormalities in mice carrying null or hypomorphic alleles of either *Sema3a*, *Ntn1*, *Ntn4*, or *Dcc* (Kagoshima and Ito, 2001; Liu et al., 2004). *Nrp1* homozygous null mice display smaller lungs with significantly fewer branches, but these observations require cautious interpretation since lung explants from these animals develop normally, suggesting the defects are a secondary effect, perhaps related to cardiovascular abnormalities (see below) (Ito et al., 2000). These negative results are inconclusive and, while unsatisfying, likely indicate the high degree of similarity in the function of these factors. If the model is correct, two Netrins and a Semaphorin function as repellents, and each could compensate for the activity of another. Thus, in the lung, functional redundancy may occur among family members (e.g., NTN1 and NTN4) and between members of functionally related families (e.g., Semaphorins and Netrins acting as repellents). This complicates the search for function, as loss of multiple cues may be required for phenotypes to be displayed. Currently, there are no descriptions of phenotypes in compound null animals in which expression of more than one of these guidance cues has been eliminated.

Other families of axon guidance cues (for example, transcripts of *Slit2* and *Slit3* and their *Robo1* and *Robo2* receptors) are expressed in the lung (Anselmo et al., 2003; Greenberg et al., 2004; Xian et al., 2001). A small

cell lung cancer line expresses a mutated *Robo1* gene termed *Dutt1*, for deleted in U2020, that is missing the first immunoglobulin repeat in the extracellular domain (Sundaresan et al., 1998). Mice engineered to express only *Dutt1* frequently die at birth due to respiratory failure, and histological analyses of their lungs reveal abnormally dense mesenchyme, surrounding smaller and irregularly shaped bronchioles (Xian et al., 2001). It is currently unknown how expression of the DUTT1 mutant results in this phenotype, although a likely explanation is that normal signals transduced by ROBO1 are perturbed, either through inappropriate interactions with SLIT, or another ROBO receptor, or an as yet unidentified cofactor. In the nervous system, SLIT signals through ROBO to promote axon branching (Ozdinler and Erzurumlu, 2002; Wang et al., 1999), therefore one possibility is that these irregularly shaped bronchioles are a result of inappropriate branching. This possibility, however, has not been directly tested, and additional data that could aid the interpretation of this phenotype, such as lung defects in other *Slit* or *Robo* null mice, have not yet been reported.

### Mammary Gland Outgrowth: Netrin-1 as an Adhesive Cue

A second organ that undergoes branching morphogenesis is the mammary gland. A unique aspect of mammary gland development is that it occurs primarily later in life, in the juvenile and adult organism, rather than the embryo. While a simple ductal structure grows prenatally, it is only after birth that the mammary tree is established by a process of ductal elongation and branching (Silberstein, 2001). Terminal end buds are the enlarged termini of ducts responsible for both growth and primary structure of the gland. Growth is driven by proliferation of a single layer of multipotent progenitor cap cells at the tip of the bud and by the underlying luminal cells. As the terminal end bud grows rapidly to the edge of the fat pad, cap cells differentiate laterally into myoepithelial cells, and a fraction drop down basally to give rise to a subpopulation of luminal cells (Williams and Daniel, 1983). Mature ducts are composed of an outer tube of myoepithelial cells that eventually contract to squeeze milk from an inner tube of secretory epithelial cells into the central lumen. Under the influence of gestational hormones, acinar lobules form along the mature ductal tree in preparation for lactation. After weaning, the lobules undergo massive apoptosis, leaving a ductal tree similar to one found in the gland prior to pregnancy.

The terminal end bud is highly invasive and motile, and the cap cells at the leading edge are also very motile. While cap cells adhere to each other through P-cadherin and luminal cells adhere to each other through E-cadherin, little was known of the mechanism that maintains the interactions between the cap and luminal cells layers. One clue recently came from expression studies showing that NTN1 is expressed by luminal cells of the terminal end bud and that its receptor NEO1, a homolog of DCC, is expressed in a complementary pattern by cap cells (Srinivasan et al., 2003). Analysis of *Ntn1*$^{-/-}$ glands reveals an inappropriate space between the cap and luminal cell layers in which loose and dying cap cells accumulate (Srinivasan et al., 2003). Loss of *Neo1* results in a similar phenotype, suggesting that NTN1 and NEO1 function as ligand and receptor in the same pathway. Together with results from in vitro assays demonstrating adhesive interactions between NTN1 and NEO1, these expression patterns and phenotypes suggest a model in which NTN1 stabilizes and maintains the close proximity of NEO1-expressing cap cells (Srinivasan et al., 2003). In this model, NTN1, secreted by luminal cells, mediates cell adhesion as a short-range attractant rather than directing cell migration as a guidance cue. This adhesive activity maintains tissue architecture during outgrowth of the gland, and it may play additional roles during the morphogenetic remodeling that occurs during pregnancy and involution.

Other cues are also expressed in the mammary gland. Similar to the expression of NTN1 and NEO1, EFNB2 is expressed by luminal cells, and its receptor, EPHB4, is expressed in a complementary pattern by myoepithelial cells (Nikolova et al., 1998). This expression pattern is dependent on estrogen and consequently regulated during the course of the estrus cycle (Nikolova et al., 1998). To examine the consequences of unscheduled EPHB4 expression on mammary gland form and function, *Ephb4* was overexpressed under the MMTV-LTR promoter, resulting in transgene expression in myoepithelial and luminal epithelial cells during pregnancy, lactation, and early involution (Munarini et al., 2002). Glands overexpressing EPHB4 display reduced proliferation, likely explaining an observed delay in their development. During pregnancy, fewer acinar lobules form in the transgenic animals, with each lobular unit containing more but smaller alveolae (Munarini et al., 2002). Inappropriate apoptotic cell death is also observed during pregnancy followed by delayed apoptosis after weaning, suggesting an overall imbalance in the response of the tissue to proliferative/apoptotic signals (Munarini et al., 2002). Since many of these phenotypes occur during stages when endogenous EFNB2 is expressed, one possibility is that perturbed receptor/ligand interactions are responsible. This, however, may be an oversimplification, as the interactions between Ephrins and their Eph receptors are known to be promiscuous (Dodelet and Pasquale, 2000), and overexpressed EPHB4 may interact with other Ephrins in the gland. Furthermore, a ligand-independent mechanism may contribute to the observed phenotypes since Eph receptor activation is one consequence of their overexpression (Zisch et al., 1997). Thus, EPHB4 overexpression interferes with the growth response of mammary epithelial cells; however, it is currently unknown whether this is a consequence of overstimulated forward signaling by EFNB2 through its overexpressed EPHB4 receptor, or unscheduled forward signaling by other Ephrins, or reverse signaling through inappropriately activated receptors.

### Cardiovascular Development: Semaphorins and Ephrins Regulate Elaborate Morphogenesis

The cardiovascular system is the first functional organ system of the vertebrate embryo. From a simple tube, a four-chambered, double pump system is generated that circulates blood separately and directionally through an ordered series of vessels. Two processes

can be distinguished during cardiovascular development: vasculogenesis and angiogenesis. In vasculogenesis, the heart primordia and primary capillary plexus, both tubular structures, develop from differentiating primitive angiogenic cells (Risau and Flamme, 1995). The heart develops when clusters of angiogenic cells coalesce to form an endocardial tube that bends, fuses, and then becomes subdivided by septa to form two atria, two ventricles, and two great vessels. The primary capillary plexus forms when a different population of angiogenic cells coalesce into a homogeneous capillary bed (Risau, 1997). This simple network of tubes is subsequently remodeled by the second process, angiogenesis, into an interconnected branched pattern characteristic of mature blood vessels. During these processes, the heart receives a critical influx of cells from the neural crest, which participate by unknown mechanisms in remodeling the great vessels, generating the endocardial cushions, and creating the adult asymmetric vasculature. Not surprisingly, members of all four families of guidance cues are expressed during cardiac development and appear to regulate various aspects of endothelial cell migration (Huminiecki et al., 2002; Lu et al., 2004; Park et al., 2003), but the most extensive studies have been on the roles of Semaphorins and Ephrins during cardiovascular development.

***Semaphorins***

In the Semaphorin family, a targeted mutation in *Sema3c* in the CD1 genetic background results in severe cardiac defects characterized by improper septation of the cardiac outflow tract (truncus arteriosus) and a ventricular septal defect (Feiner et al., 2001). Since disruption of cardiac neural crest cell migration leads to similar defects (Kirby et al., 1983), initial studies to understand the *Sema3c* homozygous null phenotypes focused on the migration of these cells (Brown et al., 2001; Feiner et al., 2001). Given that Semaphorins can function in the nervous system as long-range guidance cues, an attractive hypothesis concerning their function was that cardiac neural crest cells, which express transcripts of *Plxna2*, could be guided to the cardiac outflow tract by SEMA3C, which could be encoded by the high level of transcripts detected in this region (Brown et al., 2001). An analogous long-range guidance role has been ascribed to SEMA3A in directing hindbrain and trunk neural crest cell migration (Eickholt et al., 1999). Once recruited to this destination, it seemed reasonable that these pluripotent cells could participate in cardiovascular patterning events that lead to proper outflow tract formation. This role was proved unlikely, however, by the analysis of cardiac neural crest cells in *Sema3c*$^{-/-}$ mice (Brown et al., 2001). These cells display only a modest disruption in their location, consistent with a role for SEMA3C in modulating their final position in the outflow tract, rather than correctly guiding them to this destination. Importantly, the disruption is not sufficient to explain the severity of cardiac defects observed in *Sema3c*$^{-/-}$ mice. Furthermore, targeted disruption of a different class 3 Semaphorin, *Sema3a*, also results in cardiac defects that, like the defects observed in *Sema3c*$^{-/-}$ mice, depend on the genetic background of the mouse (Behar et al., 1996). These defects, however, are different from those observed in *Sema3c*$^{-/-}$ mice, since there are no defects observed in the outflow tract or great vessels (Behar et al., 1996; Takashima et al., 2002). Thus, neither SEMA3C nor SEMA3A appear to simply function in the cardiovascular system as a secreted, long-range guidance cue for cardiac neural crest cells. Instead, recent work to elucidate the function of class 3 Semaphorins in the cardiovascular system has focused on three different receptors expressed during cardiac development.

Neuropilins and Plexins are coreceptors for class 3 Semaphorins, but their relationship is complicated by the fact that Neuropilins also serve as coreceptors for some forms of the vascular endothelial growth factor (VEGF) family of ligands (Soker et al., 1998). In the cardiovascular system, VEGF proteins are key regulators of vasculogenesis and angiogenesis, and VEGF$^{165}$ (*Vegf*$^{165}$) likely plays overlapping roles with SEMA3C during cardiac development. This is evidenced by the phenotype of *Vegf*$^{165-/-}$ mice, which display very similar cardiac defects compared to *Sema3c*$^{-/-}$ mice (Brown et al., 2001; Stalmans et al., 2003). Analysis of mice carrying homozygous null mutations in the shared receptors for these ligands indicate that Neuropilins play a critical role in mediating signals during vascular development. *Nrp1*$^{-/-}$ mice die as embryos (E10.5–E12.5) and exhibit defects in the heart, vasculature, and nervous system (Kawasaki et al., 1999; Kitsukawa et al., 1997). In contrast, *Nrp2*$^{-/-}$ mice are viable but display defects in the nervous and lymphatic systems (Chen et al., 2000; Giger et al., 2000; Yuan et al., 2002). Double homozygous null (*Nrp1*$^{-/-}$;*Nrp2*$^{-/-}$) embryos have severe vascular defects in both embryonic and placental blood vessels, dying earlier in gestation (E8.5) compared to single *Nrp1*$^{-/-}$ mutant mice (Takashima et al., 2002). To distinguish VEGF from class 3 Semaphorin signaling through NRP1, two lines of mice were generated, one with loss of NRP1 expression only in endothelial cells (*Nrp1*$^{[endo-]}$) and the second with a mutant version of NRP1 that binds VEGF, but not SEMA3 proteins (*Nrp1*$^{[sema-]}$) (Gu et al., 2003). *Nrp1*$^{[sema-]}$ mice do not display the outflow tract and ventricular septal defects displayed in *Nrp1*$^{[endo-]}$ or *Vegf*$^{165-/-}$ animals, suggesting that normal cardiac development requires VEGF signaling through NRP1 expressed in the endothelium. In contrast, atrial enlargement, similar to that observed in *Sema3a*$^{-/-}$ mice (Behar et al., 1996), was observed in *Nrp1*$^{[sema-]}$ and *Nrp1*$^{[endo-]}$ mice, suggesting that proper atrial development requires SEMA3A/NRP1 signaling (Gu et al., 2003).

While these elegant experiments shed light on VEGF and SEMA3A signaling, the question of how SEMA3C signals during cardiac development still remained. Mice carrying homozygous mutations in both *Nrp1*$^{[sema-]}$ and *Nrp2* display outflow tract and ventricular septal defects that are not present in either single mutant animal. These defects are very similar to those displayed in *Sema3c*$^{-/-}$ mice in certain genetic backgrounds, raising the possibility that either NRP1 or NRP2 can serve as SEMA3C receptors during cardiac development, with each receptor able to compensate for the loss of the other. A breakthrough occurred with the identification of *Plxnd1*, a gene encoding a putative Neuropilin coreceptor (Gitler et al., 2004; Torres-Vazquez et al., 2004). PLXND1 is expressed in vascular endothelium (Gitler et al., 2004; Torres-Vazquez et al., 2004) and can enhance the binding of SEMA3C to NRP1 and NRP2, and SEMA3A to

NRP1 (Gitler et al., 2004). The cardiac outflow tract and ventricular septal defects in *Plxnd1*$^{-/-}$ animals are very similar to those present in *Sema3c*$^{-/-}$, *Nrp1*$^{[endo-]}$, and *Vegf*$^{165-/-}$ animals, leading the authors to suggest a model in which both SEMA3C and VEGF$^{165}$ are required for proper cardiac development (Gitler et al., 2004). In this model, SEMA3C and VEGF$^{165}$ signal through PLXND1 and the VEGF receptor, KDR, respectively, while Neuropilins serve as coreceptors for both pathways. Thus, SEMA3C may signal through PLXND1 to play a direct role in cardiac development (Gitler et al., 2004; Gu et al., 2003; Torres-Vazquez et al., 2004), rather than simply refining VEGF signaling through competitive inhibition of Neuropilin signaling (Miao et al., 1999). Certainly in zebrafish vascular development, a related class 3 Semaphorin, SEMA3A, appears to signal directly as a chemorepellent through PLXND1 to generate and organize developing blood vessels (Childs et al., 2002; Torres-Vazquez et al., 2004). Thus, current data suggest a direct role for SEMA3/PLXN signaling, coordinated with VEGF signaling, in mediating numerous aspects of cardiovascular development.

Since SEMA3C plays a critical role during cardiac development, the question of its function arises. In the developing nervous system, distinguishing attractive versus repellent roles for guidance cues such as the Semaphorins has been relatively straightforward. Expression analysis reveals the source of a cue relative to the neurons that express its receptors. In vitro collagen gel assays show whether axons, extending from tissue explants containing the cell bodies of these neurons, are attracted or repelled by the cue. These activities can then be confirmed by examining whether the axons are misguided in mice carrying homozygous null mutations in genes encoding either the cue or its receptor. In contrast, in vitro assays for cardiac development are not well developed, and while the analysis of knockout phenotypes illuminates specific defects, it is difficult to extrapolate from these defects how a specific morphogenetic event went awry. Consequently, it is much more difficult to tease out the precise activity of a guidance cue. Take, for example, the formation of the outflow tract, which is disrupted in *Sema3c*, *Nrp1*$^{[endo-]}$, *Plxnd1*, and *Vegf*$^{165}$ single homozygous null animals and in *Nrp1*$^{[sema-]}$*;Nrp2* double null homozygotes. By a number of developmental events that are still poorly understood, this bilayered tubular structure is transformed into two spiraling tubes that are capped by valves (Webb et al., 2003). While expression analysis reveals that *Sema3c* transcripts are expressed by the outer layer of cardiomyocytes and transcripts of its receptors by the inner layer of endothelial cells (Feiner et al., 2001), it is unclear how and when Semaphorin signaling occurs during outflow tract development. One clue comes from in vitro studies in which PLXND1-expressing endothelial cells are treated with SEMA3A, leading to the loss of actin stress fibers, a response associated with the loss of cell adhesion and repulsion (Torres-Vazquez et al., 2004). These data suggests that at least one class 3 Semaphorin may act at short range to disrupt adhesion between two cell layers, allowing sheets of cells to slide past one another during the remodeling that transforms this simple tissue into a complex structure. But whether destabilization of cell layers actually occurs during cardiac outflow tract formation, and whether other secreted Semaphorins, such as SEMA3C, mediate repulsion at such a close range remain open questions.

In any case, deciphering the functions of Semaphorins during cardiac development promises to be very complex as there is a plethora of Semaphorins expressed in the heart during development. Five class 3 Semaphorins and three PlexinA receptors are expressed in endothelial cells (Serini et al., 2003), and recent data suggest that one of the ways these class 3 Semaphorins function is via autocrine signals that inhibit integrin activation and decrease adhesion between cells and the ECM (Serini et al., 2003). Evidence for this type of cross-talk between integrin and guidance cue signaling is growing, not just for Semaphorins but for the other cues as well (Nakamoto et al., 2004). Elucidating how this cross-talk regulates cell-ECM interactions will be complicated by the observation that cues, such as Netrin (Yebra et al., 2003) and a class 7, GPI-linked Semaphorin (Pasterkamp et al., 2003), directly mediate integrin signaling. Furthermore, studies in chick demonstrate a bifunctional role for a transmembrane member of the class 6 Semaphorins (Toyofuku et al., 2004). SEMA6D mediates endothelial cell migration depending on which region of the heart the cells are derived (Toyofuku et al., 2004). Endothelial cells from the outflow tract are stimulated by SEMA6D, an action opposing the movement directed by SEMA3A. PLXNA1 apparently interacts in these cells with VEGF receptor type 2, allowing SEMA6D to act synergistically with VEGF to promote migration. In contrast, endothelial cells from the ventricular region of the heart are inhibited by SEMA6D, and despite the coexpression of NRP1, this inhibition is attributed to an interaction between PLXNA1 and OTK, a receptor tyrosine kinase shown in *Drosophila* to serve as a PLXNA1 coreceptor (Winberg et al., 2001). Taken together, these results describe dual roles for SEMA6D that are region specific, opposite in function and dependent on different PLXNA1/coreceptor interactions. As such, they contribute to the growing data on the elaborate ways in which Semaphorins signal through Plexins and other receptors to influence cardiac morphogenesis.

***Ephrins***

Ephrins and Eph receptors are also expressed in the cardiovascular system, with one Ephrin and its receptor expressed in a particularly striking pattern. Transcripts for *Efnb2* are expressed by arteries and not veins, and transcripts for one of its receptors, *Ephb4*, are expressed by veins and not arteries (Wang et al., 1998). The analysis of *Efnb2* and *Ephb4* homozygous null mice reveals similar phenotypes (Gerety et al., 1999; Wang et al., 1998). Early vasculogenesis in both animals appears largely normal since primitive blood vessels form, but major defects occur during angiogenesis when these vessels are remodeled into vascular networks. This shared pattern of phenotypic defects, together with the complementary expression of a ligand and receptor that signal bidirectionally, led to the hypothesis that reciprocal signaling between arteries and veins is required for remodeling the capillary network. This hypothesis was supported by initial studies on mice that express a truncated form of *Efnb2* (*Efnb2*$^{[ICD-]}$) and should therefore be incapable of reverse signaling (Adams et al., 2001). These mice display defects in angiogenesis very similar

to those observed in *Efnb2*$^{-/-}$ animals, suggesting that reverse signaling is required for angiogenic remodeling. This conclusion, however, has recently been revised with the generation and analysis of additional lines of mice (Cowan et al., 2004). One line harbors a cytoplasmic domain deletion, similar to the truncation found in *Efnb2*$^{[ICD-]}$ mice, but with the addition of a C-terminal epitope tag (*Efnb2*$^{[ICD-:HA]}$). The second line carries *Efnb2*, in which the cytoplasmic domain is replaced by β-galactosidase (*Efnb2*$^{[ICD-:LacZ]}$). Analysis of these mice shows that when the cytoplasmic domain is truncated, the protein remains trapped within the cell and is consequently not present at the cell surface to participate in signaling. In contrast, fusing the cytoplasmic domain to β-galactosidase apparently facilitates proper trafficking of the protein to the plasma membrane where it is capable of forward, but not reverse, signaling. Mice expressing this EFNB2/β-gal fusion protein display normal vascular connections, indicating that reverse signaling does not contribute to angiogenesis. These surprising new results lead to a revised hypothesis concerning EFNB2/EPHB4 signaling during angiogenesis in which EFNB2 functions solely as a traditional ligand on arteries to stimulate forward EPHB4 signaling in veins.

The discovery that only forward signaling occurs during vasculogenesis raises the question of how unidirectional signaling leads to similar arterial and venous remodeling defects in the *Efnb2* and *Ephb4* null animals. At least two models, that are not mutually exclusive, can be proposed. One model is based on the observation that EPHB activation by EFNB-Fc fusion proteins induces endothelial sprouting (Adams et al., 1999). These results are consistent with a role for forward signaling in venous remodeling, which in turn could stimulate arterial remodeling. If this is the case, arterial defects in *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mice are secondary to venous defects and are likely caused by altered or absent blood flow to veins. A second model to explain how unidirectional signaling may lead to symmetrical mutant phenotypes is based on cell mixing experiments using endothelial cells overexpressing either EPHB4 or the truncated form of Ephrin, EFNB2$^{[ICD-]}$ (Fuller et al., 2003). These cells segregate into populations favoring homotypic interactions, suggesting that forward signaling by EFNB2 through EPHB4 restricts cell intermingling by mediating repulsive rather than adhesive interactions. These results are consistent with the functional role proposed for EFNB2/EPHB4 signaling in restricting cell movement and establishing cell boundaries in adjacent hindbrain rhombomeres (Xu et al., 1999). Accordingly, this activity could establish or maintain the arterial-venous boundary at the interface between populations of endothelial cells expressing EFNB2 and EPHB4. If this is the case, then it suggests that proper boundary formation and maintenance is essential for network remodeling, and in the absence of this forward signaling neither venous nor arterial remodeling occurs.

These and other studies on *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mice, and on related members of both families, show that EFN/EPH signaling plays additional roles during cardiovascular development. For example, *Ephb2/Ephb3* double null homozygous mice display a variety of defects in vasculogenesis (Adams et al., 1999). And, even though *Efnb*$^{[ICD-:LacZ]}$ mice do not show defects in vascular remodeling, they display a variety of cardiac valve defects, indicating a role for reverse signaling during heart development (Cowan et al., 2004). Thus, similar to Semaphorin signaling in cardiovascular development, signaling by Ephrins and Eph receptors is complicated, and elucidating the roles of individual members as ligands and as receptors is hindered by the number of family members expressed and by the complexity of their actions.

**Kidney Induction: Slit2 "Unmarks" the Spot**

This review has highlighted a number of models describing the function of guidance cues during organogenesis—models that are based primarily on analogous functions of these factors in the nervous system. The complexity of organ development and the number of members of each family expressed in different tissues, however, raises the possibility that interpreting phenotypes based on known activities in the nervous system is misleading. Cues could have entirely novel functions in different contexts. Studies examining SLIT/ROBO signaling during kidney development bring this issue to the fore, with the recent analysis of *Slit2*$^{-/-}$ mice suggesting a new role for SLIT2 signaling in regulating transcription (Grieshammer et al., 2004). In contrast, efforts to demonstrate an established role for SLIT2 as a branching factor in kidney have been unsuccessful (Piper et al., 2002). While much remains to be learned about the new signaling pathway mediated by SLIT in kidney, it raises the specter of novel functions unrelated to the known branching, guidance, and adhesive activities associated with this cue.

Kidney organogenesis depends on a series of reciprocal interactions between the epithelial ureteric bud and metanephric mesenchyme. The murine kidney (metanephros) develops around embryonic day 11 when the extending nephric (Wolffian) duct produces an outgrowth called the ureteric bud, which invades a specialized region of intermediate mesoderm called the metanephric mesenchyme (Figure 3; Saxen, 1987). Signaling interactions between the ureteric bud and the metanephric mesenchyme induce the bud to grow and branch so that it arborizes into a tree-like collecting duct system. Signals from the tips of these branches are required for the formation of nephrons, the functional units of the kidney. These signals induce disorganized aggregates of mesenchymal cells to undergo a complex morphogenesis as they change shape and transition into highly organized epithelial tubules.

Ureteric bud formation is elicited by the growth factor GDNF, which is secreted by the metanephric mesenchyme and signals via its receptors expressed in the duct epithelium (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). In the absence of *Slit2*, multiple ureteric buds form, and due to a similar phenotype present in *Robo2*$^{-/-}$ mice, the data suggest that SLIT2 signals through ROBO2 to suppress supernumerary bud formation (Grieshammer et al., 2004). Interestingly, a similar phenotype is also observed as a consequence of GDNF treatment, suggesting a link between SLIT/ROBO signaling and GDNF expression (Sainio et al., 1997). Expression analysis reveals *Slit2* transcripts in the nephric duct and in the anterior nephrogenic mesenchyme, where *Robo2* is also expressed (Figure 3). In

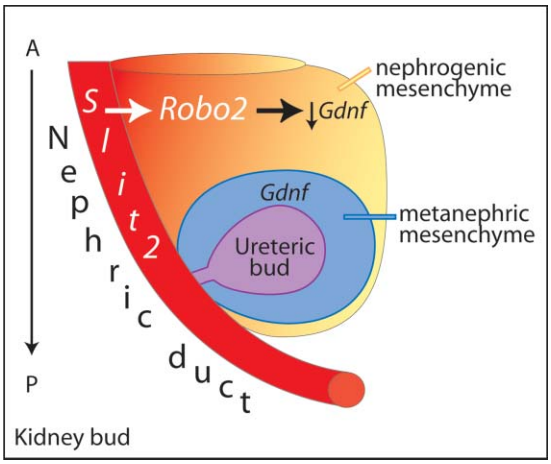


Figure 3. SLIT2 Restricts Ectopic Ureteric Buds by Signaling through ROBO2 to Inhibit *Gdnf* Expression in the Anterior Mesenchyme

*Slit2* transcripts (red gradient) are detected at a high level throughout the nephric duct and at relatively lower levels in the nephrogenic mesenchyme. In contrast, *Robo2* transcripts are expressed at a high level throughout the nephrogenic mesenchyme where *Gdnf* expression is suppressed. During development, transcripts for *Gdnf* become restricted to the posterior region where the ureteric bud forms in the metanephric mesenchyme (blue cloud surrounding the ureteric bud). These patterns of expression are consistent with a model in which SLIT2, signaling through ROBO2, restricts the anterior expression of *Gdnf*. In the posterior region, lack of ROBO2 prevents SLIT signaling, allowing *Gdnf* expression and consequently the appropriate outgrowth of the ureteric bud. Figure from Grieshammer et al. (2004).

contrast, *Gdnf* transcripts are expressed throughout the nephrogenic mesenchyme, but over the course of development they become progressively restricted to the posterior region where the ureteric bud forms (Figure 3).

Given these expression patterns and the chemorepellent activity of SLITS in the nervous system, one appealing model was that SLIT2/ROBO2 signaling guides *Gdnf*-expressing cells by repelling them from the anterior nephrogenic mesenchyme to the posterior metanephric mesenchyme, where they would be appropriately located to secrete GDNF and elicit bud outgrowth. In this model, loss of SLIT2/ROBO2 signaling would result in supernumerary bud formation since *Gdnf*-expressing cells would be present throughout the mesenchyme rather than properly restricted to the site of ureteric bud growth. Studies were performed to test this model, but it was found that SLIT2 does not appear to act as a chemorepellent for nephrogenic mesenchymal cells (Grieshammer et al., 2004). An alternate model in which SLIT2 acts to eliminate *Gdnf*-positive cells by inducing cell death was also tested. Few or no dying cells, however, were found in the nephrogenic mesenchyme of normal embryos, indicating that this mechanism is not responsible for restricting *Gdnf* expression (Grieshammer et al., 2004). As a result of these studies, the authors favor a third possible model involving the effects of SLIT2/ROBO2 signaling on *Gdnf* expression. Since GDNF elicits ureteric bud formation, SLIT2/ROBO2 signaling may function to restrict its expression in anterior nephrogenic mesenchymal cells. Consistent with this model is the observation that *Gdnf* expression is inappropriately maintained in the anterior nephrogenic mesenchyme in *Slit2* and *Robo2* null homozygotes, and reducing *Gdnf* gene dosage in *Slit2*$^{-/-}$ animals rescues the supernumerary ureteric bud phenotype.

Since these experiments do not reveal the mechanism underlying SLIT/ROBO signaling, additional studies are required. One possibility is that SLIT2 acts "in character" through ROBO2 to remodel cell contacts by modulating cell adhesion. This remodeling may indirectly affect the expression of genes by communicating with other signaling pathways, such as integrin, that directly control transcription. Alternatively, these data may reflect a new role for SLIT/ROBO signaling in directly regulating, through as yet uncharacterized transduction pathways, the transcription or translation of genes encoding crucial morphogenetic cues. The idea that ROBO signaling may regulate levels of signaling proteins is supported by recent studies in *Drosophila*. SLIT acts through ROBO to promote terminal asymmetric division of ganglion mother cells by downregulating the expression of two POU proteins, Nubbin and Mitimere, and consequently allowing the asymmetric localization of Inscuteable (Mehta and Bhat, 2001). In a different context, ROBO signaling in a SLIT-independent manner is required for serotonergic neuron differentiation by regulating the expression of a transcription factor, Eagle, required for this process (Couch et al., 2004). While additional studies are required to delineate these pathways, evidence is accumulating that SLIT/ROBO- and ROBO-mediated signaling does more than just reorganize the cytoskeleton to direct cell motility. Indeed, it appears it may have broad-ranging roles in regulating the transcription and/or translation of key factors that specify cell fates.

## Future Directions

Cells interact with each other in diverse ways to form complex structures during organogenesis. Recent studies analyzing the biological roles of axon guidance cues outside the nervous system demonstrate that these multifunctional cues play diverse roles that profoundly influence the generation of complex tissues. Studies on the function of these molecules in the nervous system provide valuable conceptual clues about their function in other contexts. In general, guidance cues shape the architecture of organs. In the nervous system, cues direct the construction of elaborate networks of connections by guiding neurons and their axonal and dendritic projections. In the heart, mammary gland, and lung, cues direct the organization of tissues by regulating cellular interactions. These processes rely on changes in cell-cell or cell-ECM contact and likely occur through transduction pathways similar to those used in axon guidance. These pathways lead to cytoskeletal rearrangements that, in turn, direct cell motility and adhesion. In contrast, studies in kidney and *Drosophila* on ROBO signaling highlight novel ways for cues to control organ architecture by regulating the expression of crucial morphogenetic factors. Such a role for "axon guidance" molecules expands our notion of their function. As such, it contributes to our broader understanding as biologists of the ways in which relatively few proteins perform wide-ranging functions that depend on in vivo context to provide the regulation required to generate an organism.

The next step for developmental biologists is to define the precise activities of these cues and to elucidate their signaling mechanisms. As outlined in this review, this will require increasingly sophisticated genetic manipulations. For example, in the lung, the multiplicity of cues that act in functionally related ways has made it impossible to verify their activity by analyzing animals harboring a deletion in a single gene encoding a cue. Consequently, deciphering the actions of cues that function redundantly will require generating compound homozygous null animals to eliminate the expression of more than one member of a single family and multiple members of different, functionally related families. A second complication in studying these cues in organs other than the nervous system is that their signaling pathways intersect with other pathways, and in some cases these shared pathways play critical roles in organ development. The example of Semaphorin/VEGF signaling through their Neuropilin coreceptor during cardiovascular development is presented in this review. Distinguishing between the two pathways required structure/function analysis of Neuropilin followed by the generation of mice expressing a functionally simplified receptor that binds VEGF but not Semaphorin. A second example is bidirectional signaling via transmembrane Ephrin and Eph receptor. To distinguish the operative signaling pathway through these proteins required generating mice that express receptors with cytoplasmic domain truncations, forcing unidirectional signaling. Developmental biologists must undertake more of these state-of-the-art transgenic manipulations to resolve specific signaling pathways during organogenesis. The analysis of these transgenic mice will greatly benefit from advances in high-resolution imaging of organ cultures and whole animals. Together, these investigations promise to reveal the critical ways in which guidance cues mediate cellular interactions as tissues undergo complex morphogenetic changes.

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